The Negative Regulation of Red Cell Mass by Neocytolysis: Physiologic and Pathophysiologic Manifestations

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Key Words

Erythropoietin • Red Cell Mass • Hemolytic Anemia • Neocytolysis • Erythropoiesis • Spaceflight Anemia • Blood Doping • Polycythemia

Abstract

We have uncovered a physiologic process which negatively regulates the red cell mass by selectively hemolyzing young circulating red blood cells. This allows fine control of the number of circulating red blood cells under steady-state conditions and relatively rapid adaptation to new environments. Neocytolysis is initiated by a fall in erythropoietin levels, so this hormone remains the major regulator of red cell mass both with anemia and with red cell excess. Physiologic situations in which there is increased neocytolysis include the emergence of newborns from the hypoxic uterine environment and the descent of polycythemic high-altitude dwellers to sea level. The process first became apparent while investigating the mechanism of the anemia that invariably occurs after spaceflight. Astronauts experience acute central plethora on entering microgravity resulting in erythropoietin suppression and neocytolysis, but the reduced blood volume and red cell mass become suddenly maladaptive on re-

entry to earth's gravity. The pathologic erythropoietin deficiency of renal disease precipitates neocytolysis, which explains the prolongation of red cell survival consistently resulting from erythropoietin therapy and points to optimally efficient erythropoietin dosing schedules. Implications should extend to a number of other physiologic and pathologic situations including polycythemias, hemolytic anemias, "blood-doping" by elite athletes, and oxygen therapy. It is likely that erythropoietin influences endothelial cells which in turn signal reticuloendothelial phagocytes to destroy or permit the survival of young red cells marked by surface molecules. Ongoing studies to identify the molecular targets and cytokine intermediaries should facilitate detection, dissection and eventual therapeutic manipulation of the process.

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Neocytolysis is a physiologic process that aids in the control of red cell mass by down-regulating it when it is excessive [1]. It is precipitated by erythropoietin (EPO) suppression, which in turn leads to the selective hemolysis of the youngest circulating red blood cells (neocytes),

allowing rapid and efficient adaptation. The process first became apparent through investigations of the mechanism of anemia that invariably afflicts astronauts returning from spaceflight [1, 2]. Neocytolysis and the role of EPO suppression have been confirmed in a variety of physiologic and pathophysiologic situations including the descent of polycythemic high altitude dwellers to sea level [3], the anemia of renal failure [4], and in a human model based on exogenous EPO administration then withdrawal (Rice L, Alfrey CP, unpublished data). We believe that low levels of neocytolysis normally function to maintain the red cell mass at an optimal level, and the level increases rapidly in response to environmental changes. Implications are broad and should extend to a number of both common and arcane situations, some of which include polycythemias, hemolytic anemias, oxygen therapy, and complications and testing for surreptitious “blood-doping” by endurance athletes. In this report, we review evidence supporting the existence of neocytolysis, some of the implications, the status of investigations into underlying mechanisms and directions for future research.

Discovery of the process in space

Astronauts on space missions of more than three days invariably lose 10-15% of their red cell mass detectible on their return to earth. This problem is not benign, but is likely a major factor to the weakness and orthostatic intolerance experienced by many returning astronauts [5]. The mechanism of spaceflight anemia defied explanation for decades but was unraveled from studies of six astronauts on the SLS-1 and SLS-2 missions [1, 2, 6]. On entering microgravity within minutes of launch, the 20% of blood volume normally held in the extremities by gravity is released to pool centrally. Within 24 hours, the body adapts by transudating of 20% of the plasma volume into the soft tissues and suppressing EPO secretion. Ten to 15% of red cells are lost over the next few days. Radiolabeled iron injected one to three days into spaceflight appears normally in newly-made red cells, demonstrating that there is no decrement in red cell production during this time when red cell mass is falling. Red cells that are labeled with Cr51 12 days or 14 days prior to launch survive normally; in fact, a very consistent “hump” in the red cell survival curve makes it appear that labeled red cells survive longer than normal (Figure 1). There is only one plausible explanation for the apparent paradox that red cell mass falls as both red cell production and survival proceed normally. A large proportion of young circulating red blood cells, those made in the 12-14 days before launch and thus not bearing the Cr51 label, must

Fig. 1. Red cell survival on SLS-2 space mission. The points are a composite of data from three studied astronauts (2,26). Red cell survival is normal pre-launch, then appears “super-normal” post-launch due to the destruction of unlabeled neocytes. The last point is based on measured chromium remaining, corrected for the red cell mass measured on landing day. The fact that the trend line generated from preflight values transects this point demonstrates that older labeled red cells are removed from the circulation at the same normal rate in space as on earth.

be selectively hemolyzed. This also explains the “hump” in the red cell survival curve—the labeled cells are less diluted by unlabeled neocytes that have been destroyed.

The theory emerges

EPO had been held to be the sole physiologic regulator of red cell mass by preventing the apoptosis of early bone marrow red cell progenitors [7]. EPO thus permits proliferation, maturation and increased red cell output at times when red cell number is low (anemia and/or hypoxia). When red cell mass becomes too high, EPO secretion is suppressed. Erythroid progenitors then undergo increased apoptosis and very slowly the red cell mass declines. If this were the only control over red cell mass, the ability of the body to control it at an optimum level would be coarse, and the ability to adapt to situations of red cell excess would be interminably slow. Because EPO suppression does not affect the maturation of committed erythroid precursors, there would be no decrement in red cell output for one week after EPO levels decline. Thereafter, red cell mass would fall by less than 1% per day (given the 120 day survival of red cells). One situation where more rapid adaptation would be desirable and might exert evolutionary pressures is the neonatal period. Newborns emerge polycythemic from

the hypoxic intrauterine environment, but at birth their high hematocrits are maladaptive for optimizing survival prospects in their new ambient air environment.

Based on the observations on spaceflight anemia, we hypothesized that EPO continues to be the main regulator of red cell mass in times of excess. When levels are suppressed, not only are there effects on red cell progenitors which eventuate in diminished red cell production and a slow decline in red cell mass, but there are also changes in the interactions between reticuloendothelial macrophages and young circulating red cells that lead to the destruction of the latter. This produces a limited, efficient and rapid adaptation. This theory of both positive and negative regulatory roles for EPO over red blood cells continues an emerging biologic paradigm that many cells depend on growth factors both for their production and for their continued survival. Red blood cells are non-nucleated (do not contain DNA) and therefore cannot undergo apoptosis, so neocytolysis has arisen as a complementary process.

While on first impression, the theory appears to fly in the face of long-held dogmas in hematology, it actually may be very consistent with seminal observations on erythropoiesis made a half century ago. One dogma has been that red cells survive 120 days in the circulation and no physiologic mechanisms can shorten this. Another dogma was that EPO actions are highly specific for red cell progenitors, but it is now widely accepted that many cell types bear receptors for and respond to EPO, particularly endothelial cells [8, 9]. Stohlman's studies of red cell incorporation of radiolabeled iron more than 50 years ago concluded that some degree of ineffective erythropoiesis was normal; this could be abrogated by EPO-rich plasma or exacerbated by EPO-deficient plasma [10]. The selective destruction of young red blood cells would be even more consistent with his observations because the apoptosis of progenitor cells (where we now know EPO works) should not substantially contribute to a ferrokinetic pattern of ineffective erythropoiesis, being that progenitors take up negligible amounts of iron, but neocytolysis would produce such a pattern. Neocytolysis can also reconcile for the first time observations made in 1950's on descent from high altitude (see below).

If neocytolysis exists, then its footprints should be discernible in a number of physiologic and pathophysiologic situations beyond spaceflight anemia and beyond the adaptation of polycythemic newborns. Neocytolysis should occur when dwellers at high altitude descend to sea level. The polycythemia generated in response to the hypoxic high altitude environment becomes suddenly maladaptive

and their situation would be analogous to that of astronauts. Additionally, neocytolysis should contribute to the anemia of renal disease, a disorder of pathological EPO deficiency. There should be implications on blood-doping by endurance athletes, because the supraphysiologic red cell mass created by transient use of erythropoietic hormones or red blood cell transfusion should suppress endogenous EPO and precipitate neocytolysis. In an effort to prove the existence of neocytolysis, we studied descending altitude dwellers, renal failure patients and blood-doping.

Neocytolysis and the role of EPO confirmed on descent from high altitude

Studies done more than fifty years ago in the Peruvian Andes and Himalayas looked at residents at very high altitude who rapidly descended to sea level [11, 12, 13]. Merino found that the red cell mass fell 10-18% in one week accompanied by increases in serum unconjugated bilirubin and fecal urobilinogen, concluding that "a very distinct hemolytic process occurred in all cases" [11]. Huff reached identical conclusions after performing ferrokinetic studies, reticulocyte counts and bone marrow exams on descent from altitude—while the red cell mass fell 9% in the first week, red cell production did not decline until 8-10 days at sea level [12]. The delay before red cell production declines is predicted from EPO's actions on early red cell progenitors but not on committed precursors. The observed hemolysis cannot be explained by tenets that no physiologic mechanisms exist to shorten red cell survival when red cell mass is excessive. These observations have long been ignored and forgotten, perhaps largely because they did not fit prevailing paradigms on the control of red cell mass. What is new about neocytolysis is that the young red blood cells are uniquely susceptible to this hemolytic process, allowing more rapid and efficient adaptation.

We studied nine inhabitants of Cerro de Pasco, Peru, living at 14,500 feet, with baseline hematocrits of 65-75%. On descent to sea level (a six hour drive to Lima), the predicted EPO suppression and red cell decline occurred, along with evidence from differential isotope labeling studies that the young red blood cells had been selectively hemolyzed [3]. As predicted by the effects of EPO suppression on progenitor cells and as Huff had observed [12], reticulocyte egress from the marrow proceeded normally while red cell mass fell by 10%; reticulocyte count diminished only after seven days at sea level. In three subjects, low doses of EPO administered on descent completely abrogated neocytolysis. Thus, the theory of neocytolysis and a probable role for EPO were confirmed.

Fig. 2. Changes in serum ferritin in three healthy subjects with EPO administration and after stopping EPO. The ferritin rose rapidly toward baseline in all subjects as neocytes were hemolyzed and their iron transferred to stores (Rice L, Alfrey CP, unpublished data).

Neocytolysis and renal disease

Dialysis patients predictably suffer anemia, often severe, which is attributable to the inability of their diseased kidneys to manufacture EPO. EPO therapy is extremely effective in correcting this anemia. Now that such therapy is routine for renal-impaired patients, it has been forgotten that renal anemia is not just due to a red cell production problem, but studies consistently demonstrated a hemolytic component of variable severity [14]. This hemolytic component has never been satisfactorily explained. With EPO therapy, numerous studies now document prolongation of red cell survival in renal patients [15, 16], another phenomenon unexplainable by prevailing dogmas but predicted by neocytolysis.

We withheld EPO therapy from stable dialysis patients and performed isotope measurements of red cell survival and stool porphyrin excretion (the breakdown products of red cell hemoglobin) [4]. We attempted to differentially label young and old cohorts of red cells by administering ^{14}C -glycine and ^{15}N -glycine two weeks apart. A pattern suggestive of neocytolysis emerged. Others have confirmed the occurrence of neocytolysis in patients with end-stage renal disease [17]. Neocytolysis can explain the well-documented superior efficiency of subcutaneous EPO over intravenous boluses [18, 19], as subcutaneous therapy can better sustain serum hormone levels above the threshold needed to precipitate the process. Thus, understanding neocytolysis could help establish optimally cost-effective dosing schedules, and may help explain some side-effects of EPO in renal patients, such as increased potassium levels.

Applicability to other physiologic and pathophysiologic situations

Unrecognized neocytolysis may be a factor in a number of both mundane and arcane clinical situations.

First, any physiologic suppression of EPO should precipitate neocytolysis. This could apply to hospitalized patients given oxygen therapy and might explain some of its untoward effects [20]. Second, pathologic suppression of EPO could produce neocytolysis which is deleterious, not adaptive. For example, the very low EPO levels in premature infants could precipitate neocytolysis that contributes to dangerous neonatal jaundice. There are, no doubt, yet undefined situations in which the neocytolytic mechanism is perturbed apart from changes in EPO. One such situation might possibly be congenital pyruvate kinase deficiency, a disorder in which young red blood cells are selectively hemolyzed but explanations have never been well-supported. When the underlying mechanisms of neocytolysis are better understood, new therapeutic approaches might be realizable for diverse problems such as polycythemia (with polycythemia vera, erythrocytes must have mechanisms to escape the process), hemolytic anemias and neonatal jaundice.

Another situation in which neocytolysis should come into play is blood-doping. Endurance athletes attempt to gain a competitive advantage by the surreptitious administration of erythropoietic hormones (EPO, darbepoietin) or by supraphysiologic red cell transfusions. Erythropoietic hormones are stopped before competition to defy direct detection in blood or urine, so either method of blood-doping should predictably suppress endogenous EPO and induce neocytolysis. This may contribute to serious health consequences that have afflicted blood-doping athletes [21]. It offers an alternative explanation for the results of an auto-transfusion study which found a lesser than anticipated increase in hemoglobin concentration coupled with an increase in serum bilirubin, findings that the authors speculated might be due to improper blood storage [22]. In unpublished experiments to establish a human model of neocytolysis, three

Fig. 3. Proposed model of neocytolysis. Endothelial cells respond to changes in circulating EPO by influencing the interaction of phagocytes with young red blood cells, the latter targeted by surface adhesion molecules [25].

volunteers self-injected EPO for one month, then suddenly stopped injections, in effect duplicating the procedures of blood-doping athletes (Rice L, Alfrey CP, unpublished data). All three experienced a rapid fall in red blood cell mass toward their baseline on EPO discontinuation. This was most clear from rapid changes in serum ferritin as the iron that had been circulating in red cells was rapidly transferred to reticuloendothelial stores (Figure 2). Ferritin had been shown to be an excellent surrogate marker for neocytolysis in altitude and spaceflight studies, having an advantage over hemoglobin and hematocrit determinations in that there is little effect of concomitant changes in plasma volume [3]. As blood-doping should very reliably precipitate neocytolysis, identifying the molecular underpinnings and markers for the process should permit sensitive, reproducible detection tests.

Molecular mechanisms of neocytolysis

It is known that a variety of red blood cell adhesion molecules exist on the surface of marrow red cell progenitors and that these undergo changes with differentiation and maturation. These surface adhesion molecules are important in the maturation process and the compartmentalization of cells in the marrow, controlling their eventual release into the circulation. Mature circulating red cells generally lack these adhesion molecules. It seems likely that the youngest circulating red cells maintain surface adhesion molecule expression to some extent; there ought to be differential expression of these molecules between young and old red cells. Young circulating red cells must interact intimately with reticuloendothelial phagocytes, particularly in the spleen, having their membranes modeled, de-pitted, and cytoplasmic inclusions culled such as Howell-Jolly bodies

and Pappenheimer bodies [23]. It seems likely that surface adhesion molecules, which can mediate phagocyte interactions, are the targets that mark young red cells for neocytolysis.

A variety of red cell adhesion molecules have been identified as candidate targets, or at least markers, for neocytolysis. Plans are to study a panel of red cell surface molecules including CD35, CD36, CD44 (HCAM), CD47, CD49e (VLA4), CD55 (DAF), cadherin, von Willebrand factor, glycophorin A, ICAM4, chondroitin sulfate and phosphatidyl serine. A serious obstacle to research on neocytolysis has been the difficulty in separating and identifying young red blood cells from older ones; there are no established, simple, reliable or reproducible methods. Flow cytometric assays for adhesion molecules on circulating red cells should be able to selectively identify young red cells and could prove a powerful tool. One application could be to detect blood-doping by athletes. It is likely that among the identified markers would be found the actual target for destruction by reticuloendothelial cells when EPO levels fall, a finding that would aid in the possible therapeutic manipulation of the process.

Neither circulating red cells nor reticuloendothelial phagocytes have EPO receptors, so they cannot respond directly to changes in circulating EPO. Endothelial cells do possess EPO receptors and respond to hormone levels in a variety of ways. EPO regulates endothelial secretion of angiogenic factors (VEGF) and complement inhibitors [8]. In an *in vitro* model of neocytolysis using cell chambers, Trial demonstrated that splenic endothelial cells respond to EPO withdrawal in unique ways (compared to other endothelial cells): they undergo distinct morphologic changes on electron microscopy, become selectively more permeable to young red blood cells, and

permit increased phagocytosis of the young red cells by macrophages [9]. EPO may regulate endothelial cell secretion of transforming growth factor- β which could influence macrophage phagocytosis [24]. The most logical mechanism of neocytolysis would involve EPO influencing endothelial cell secretory responses that in turn influence the interaction of neocytes with reticuloendothelial cells, determining whether the cell continues to circulate or is instead phagocytized and lysed (Figure 3).

Future directions

Ample evidence demonstrates the reality of neocytolysis, a physiologic process that helps regulate the normal red cell mass by selectively hemolyzing young circulating red cells in response to lowered EPO levels. It further facilitates rapid adaptation to new environments, and it bears on a number of pathophysiologic conditions.

The identification of red cell surface molecules which are markers or targets for neocytolysis should facilitate progress in understanding the process, overcoming the hindrance that there are not presently reliable methods to distinguish young red cells from old. Questions can be addressed in human models (such as the EPO administration model discussed above) on the limitations of the process: how low must EPO levels fall, for what period of time, etc. Establishment of rodent models is under consideration. With improved methods of detection, the role of neocytolysis in polycythemic disorders, hemolytic disorders, oxygen toxicity and other clinical situations can be elucidated. Therapeutic manipulation of the process should be feasible. It is the authors' hope to excite more investigators on the prospects of this newly-discovered process, expediting scientific and clinical advances.

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